OPIC
Office de la propriété intellectuelle du Canada

CIPO
Canadian Intellectual Property Office

(12) (19) (CA) **Demande-Application**

(21) (A1) **2,225,866**
(22) 1997/12/24
(43) 1999/06/24

(72) GUSTAFSON, Stefan, SE
(71) HYAL PHARMACEUTICAL CORPORATION, CA

(51) Int.Cl.[6] C08B 37/00

(54) **PURIFICATION DE POLYSACCHARIDES ET PRODUITS ULTRA PURS AINSI OBTENUS**

(54) **PURIFICATION OF POLYSACCHARIDES AND ULTRA PURE PRODUCTS RESULTING THEREFROM**

(57) This invention relates to the purification of polysaccharides, for example hyaluronan of contaminants, for example proteins, and in the case of protein, a method of purifying hyaluronan to a protein content no greater than about 0.03%.

ABSTRACT

This invention relates to the purification of polysaccharides, for example hyaluronan of contaminants, for example proteins, and in the case of protein, a method of purifying hyaluronan to a protein content no greater than about 0.03%.

TITLE OF INVENTION

Purification of Polysaccharides and Ultra Pure Products Resulting Therefrom.

FIELD OF INVENTION

This invention relates to the purification of polysaccharides (e.g. hyaluronan, chondroitin sulphate, etc.) of contaminants (e.g. proteins) in the pharmaceutical field or therapeutic field or the like, by the adsorption of the contaminants (e.g. proteins) to a medium which attracts said contaminants (e.g. proteins) from the polysaccharide (e.g. hyaluronan) resulting in a polysaccharide (e.g. hyaluronan) having a lower amount of contaminant (e.g. protein), and thus resulting in an ultrapurified polysaccharide (e.g. hyaluronan).

BACKGROUND OF INVENTION

Schmut et al., Biochimica et Biophysica Acta, 673 (1981), 192-196, purport that isolation of purified hyaluronic acid from a vitreous body, may be achieved by precipitation with cetyl pyridium chloride, Dowex 50 cation-exchange resin, and DEAE-Sephadex have been used. Proteolytic enzymes such as papain are now commonly used for the isolation of pure hyaluronic acid; however, even after repeated proteolysis, hyaluronic acid is always associated with a small amount of protein. The bulk of the contaminant proteins was removed by treatment with cold trichloroacetic acid. Schmut et al. purport to have arrived at a hyaluronic acid having a protein content of 0.3%, by the treatment of hyaluronic acid with activated CH-Sepharose 4B to arrive at that concentration. Schmut et al. further purport that the best purification of the hyaluronic acid, however, was achieved by a combination of Dowex 1-X2 column chromatography and treatment with activated CH-Sepharose 4B. Trichloroacetic acid treated hyaluronic acid was at first purified by Dowex 1-X2 column chromatography, where the 0.5-0.65 M NaCl fraction yielded a protein-poor hyaluronic acid which was free of other glycosaminoglycans. By two-fold treatment of this hyaluronic acid preparation with activated CH-Sepharose 4B, it was purported that a bovine vitreous body

hyaluronic acid with a protein content of about 0.1% was obtained as determined by the method of Lowry et al. (1951) J. Biol. Chem., 193, 265-275.

Radaeva et al. 1995, Russian Biotechnology No. 12, pp. 40-44, purport that proteins from hyaluronic acid may be removed by denaturation or by further enzymatic conversion, and a mixture of glycosaminoglycans may be separated by fractional precipitation with cetavlon or by ion exchange chromatography. The purification of hyaluronic acid from proteins is performed by extraction with chloroform with amyl alcohol, by extraction with phenol, by enzymatic hydrolysis with papain, by precipitation with cetylpyridinium chloride, by adsorption on activated charcoal, by electrodialysis, or by extraction with sodium acetate. There is no discussion in Radaeva et al. as to the resultant protein concentration in the purified hyaluronic acid after undergoing any of the above mentioned methods.

Balazs (U.S. Patent 4,141,973) purports that a hyaluronic acid fraction after undergoing extraction and purification results in a protein content of less than 0.5% by weight. This extraction is performed with the use of chloroform under acidic conditions.

The above references indicate that the purification techniques resulted in a hyaluronan having a protein content at best 0.1% and the purification was carried out under acidic conditions. Furthermore, in previous studies of methods of adsorbing hyaluronic acid to plastic dishes, the presence of hyaluronic acid on the dishes was never directly shown (Goetinck et al. (1987), J. Cell Biology, 105:2403-2408) and when Applicant tried to repeat some of the earlier studies, Applicant found that hyaluronic acid does not bind to plastic but that the protein contaminants do. Thus, in light of my discovery that protein contaminants adsorb to the plastic, a method has now been devised by me to ultra-purify known preparations of hyaluronic acid and other negatively charged polysaccharides.

SUMMARY OF INVENTION

Applicant has now discovered a process of purifying polysaccharides of contaminants such that the contaminants content is no greater than about 0.09% and preferably less than about 0.03%. The purification process is carried out by the introduction of a polysaccharide to a medium (e.g. plastic) which attracts the contaminant. The introduction is carried out for a sufficient period of time to result in a reduced contaminant content in the polysaccharide.

It is therefore an object of this invention to provide a highly purified polysaccharide substantially free of contaminants.

It is also another object of this invention to provide a method of purifying hyaluronan of protein content such that the resultant hyaluronan has a protein content no greater than about 0.09%, and preferably no greater than about 0.03% and still most preferably less than about 0.03%.

It is yet another object of the invention to provide an ultra-purified form of hyaluronan having a protein content no greater than about 0.03% and still most preferably less than about 0.03%.

It is yet another object of the invention to provide an ultra-purified form of hyaluronan having a protein content no greater than about 0.03% and still most preferably less than about 0.03% whenever produced by contacting hyaluronan having a protein content greater than about 0.09% to a medium, preferably plastic, for a sufficient period of time to result in said ultra-purified form of hyaluronan.

It is therefore an object of the invention to provide a process of purifying a polysaccharide, said process comprising introducing the polysaccharide which contains contaminants onto a medium, which medium attracts the contaminants thereto, for a predetermined period of time; and recovering the contaminant reduced polysaccharide.

In a preferred embodiment, before recovering the contaminant reduced polysaccharide which comprises transferring the contaminant reduced polysaccharide from the medium after said predetermined period of time and introducing said contaminant reduced polysaccharide from the medium to a second medium which second medium attracts contaminants and continuing said process until the desired degree of purification is achieved.

In a preferred embodiment, said medium comprises a substantially negatively charged medium, preferably plastic or the like.

In even yet another preferred embodiment, said plastic is a plurality of plastic beads or the like.

Preferably the above process is carried out at substantially physiological pH.

Preferably the above process is carried out at substantially physiological ionic strength.

In a preferred embodiment, the polysaccharide, preferably a glycosaminoglycan, preferably hyaluronan or chondroitin sulphate.

It is therefore another object of the invention to provide a highly purified form of a polysaccharide, preferably hyaluronan or chondroitin sulphate, having a protein content no greater than about 0.09% by weight, preferably said protein content being no greater than about 0.03% by weight.

It is yet another object of the invention to provide a purified form of a polysaccharide, preferably hyaluronan or chondroitin sulphate, having a protein content no greater than about 0.09% by weight preferably no greater than about 0.03% by weight wherein produced by introducing said polysaccharide onto a medium, preferably a substantially negatively charged medium, preferably plastic, plastic beads or the like, for a predetermined period of time, and recovering the protein reduced polysaccharide.

In a preferred embodiment said protein reduced polysaccharide is transferred from the medium after said predetermined period of time and introducing said

protein reduced polysaccharide from the medium to a second medium for a predetermined period of time until the desired degree of purification is achieved. Further and other objects of the invention will become apparent to a person skilled in the art in view of the following examples:

BRIEF DESCRIPTION OF THE DRAWINGS

The following figures illustrate preferred and alternative embodiments of the invention, wherein:

Figure 1: Identification of HA using a specific ^{125}I-HABP. Wells coated with the indicated substances and in the case of bovine serum albumine (BSA), polylysine (poly-l) and a synthetic peptide (ICAM) also S-HA, or untreated wells (C) received after washing ^{125}I-HABP (approximately 7000 cpm). After 1h incubation the wells were washed and the radioactivity in the wells measured.

Figure 2: Binding of ^{125}I-S-HY (approximately 25ng/60µl, 200,000 cpm) to ELISA-plates in the absence (C) or presence of hUC-HA or S-HA for 16h at room temperature. After washing the radioactivity associated with the wells was extracted and measured.

Figure 3: SDS-PAGE of material from two HA preparations with affinity for plastic. 50µl of hUC-HA or S-HA were incubated with ELISA-plates over night. After extensive washing, 40 µl of electrophoresis sample buffer containing SDS and DTT was consecutively added to 5 wells and the material electrophoresed and stained as described in the Materials and Methods section. Immunoblotting of adsorbed hUC-HA material using polyclonal antibodies to fibronectin (hUC-HA IB) shows staining at the top of the gel, at 220-240 kDa and non specific staining at the bottom of the gel. The position of high molecular weight standard proteins are indicated.

MATERIALS AND METHODS:

Polysaccharides: HA from human umbilical cord (hUC-HA), chondroitin sulphate C from shark cartilage (CSC), chondroitin sulphate A from bovine

trachea (CSA) and a HA preparation produced by streptococci (S-HA) were obtained from Sigma Chemical Co. Rooster comb HA (RC-HA) was from Pharmacia & Upjohn, Uppsala, Sweden. A streptococcal HA (S-HY) was supplied by Hyal Pharmaceutical Corporation, Toronto, Canada. The S-HY was also used for ^{125}I-labelling (Gustafson et al, 1994, Glycoconjugate J. 11:608-613). The specific radioactivity was between 700 and 4000 dpm/ng.

Plastic Plates: Well plates are available in polystyrene and polypropylene composition, for example, Greiner America markets microplates of polystyrene and polypropylene composition. The applicant has used ELISA-plates from Flow laboratories (Titertek) and also wells and plates for cell culture such as Nunclon (Nunc AS, Denmark) and Falcon (Becton Dickinson, New Jersey). However, any suitable "active" plastic surface can be used herein. "Active" is known by persons skilled in the art to mean electrically charged.

Proteins and peptides: Human fibronectin was a kind gift from Professor K. Rubin at Applicant's department at the University of Uppsala in Sweden. It was judged >99% pure by SDS polyacrylamide gel electrophoresis (SDS-PAGE).

An amino acid sequence found in the proposed intracellular part of ICAM-1, RQRKIRIYKLQKA, was from Peptide-lab, Department of Medical and Physiological Chemistry, Uppsala, Sweden. It was purified by reversed phase chromatography and judged pure by mass spectrometry.

Polylysine was obtained from Sigma Chemical Company.

Radiolabeled specific hyaluronan binding protein from cartilage (^{125}I-HABP) was obtained from Pharmacia & Upjohn, Uppsala, Sweden (as a part of the HA-50 test).

Protein Determination: Protein was determined using a miniaturized version of the method of Bradford (1976) using BSA as standard: 50-100 µl of sample or protein standards were added to microtiter wells and 100-200 µl of reagent added.

The absorbance at 600 nm correlated well with the protein standards and different dilutions of the samples.

SDS-gel electrophoresis and immunoblotting: Samples of hUC-HA, S-HA, RC-HA, S-HY, ^{125}I-S-HY, human fibronectin and molecular weight standards (Bio-Rad) adsorbed to plastic (16h), or dissolved in PBS were electrophoresed on 5% or 10% polyacrylamide gels in the presence of SDS. Before electrophoresis some samples were reduced with dithiotreitol. After electrophoresis the proteins were visualized using silver staining and radioactivity visualized using phosphoimaging (Gustafson and Björkman, 1997, Glycoconjugate J. In press) after drying of the gels. In some cases the gels were analyzed by immunoblotting using the polyclonal antibody to human fibronectin (1:10000) after transfer of the proteins to nitrocellulose. Detection was performed using peroxidase labelled goat anti rabbit IgG (1:10000; Vector) and chemiluminiscense as described previously (Forsberg et al, 1994).

Detection of HA on plastic coated with HA preparations or peptides:
The HA preparations (2.5 mg/ml) and bovine serum albumine (1mg/ml) were allowed to bind to the plates for 1h, the polylysine or synthetic peptide (20µg/ml) were allowed to bind 30 min whereafter S-HA (2.5 mg/ml) was added and incubated for the remaining 30 min. After 5 washes with 200 µl PBS, 90 µl ^{125}I-HABP, diluted 1:150 in PBS, was added and allowed to bind for 1h. After 4 washes with 200 µl PBS, the bound radioactivity was extracted with lysis buffer as described earlier (Gustafson et al, 1994 idem) and measured on a Packard Autogamma gammacounter.

Binding of labelled HA to plastic: 60 µl ^{125}I-S-HY in PBS or hUC-HA (2.5 mg/ml in PBS) or S-HA (2.5 mg/ml in PBS) was added to ELISA plates and allowed to bind for 2 or 16h. After binding the plates were washed with PBS 5x300 µl and the radioactivity extracted with lysis buffer and analyzed as described above.

Binding of HA to fibronectin coated wells: 60 µl fibronectin (10µg/ml) was added to each well and allowed to bind for 16h. After washing with PBS, 50µl of ^{125}I-S-HY was added to each well and allowed to interact for 1-2h. After washing the radioactivity associated with each well was determined by extraction with lysis buffer as described above.

Immunological detection of fibronectin: A rabbit monospecific polyclonal antibody to human fibronectin was kindly supplied by Professor K. Rubin at my Department, at Uppsala University, Uppsala, Sweden.

ELISA plates were coated with human fibronectin (50 µl, 10 µg/ml) and remaining binding sites blocked by addition of 50 µl 2.5% BSA. After washes with PBS containing 0.05% Tween 20, Fibronectin or hUC-HA was added at different dilutions and the primary antibody added at 1:10000 dilution for 1h, the plates washed and the secondary peroxidase labeled goat antirabbit antibody (Vector laboratories) added at 1:10000 dilution. After 30 min incubation the plates were washed and the enzyme detected with orthophenyldiamine+H_2O_2. The optical density (OD) at 450 nm was read on an automatic ELISA reader (Labsystems, Multiskan MS). In some cases the plates were coated with hUC-HA or S-HA instead of human fibronectin and the primary antibody or preimmune serum (from the same animal) added without competitors in a direct ELISA.

Results

When the presence of HA on ELISA plates coated with hUC-HA, S-HA,. BSA, ICAM-1 peptide or polylysine, and where BSA and the peptides were additionally allowed to interact with S-HA, the specific ^{125}I-labelled HABP could only detect HA on the peptides carrying an abundance of positively charged amino acids (Fig 1).

When 250 ng of ^{125}I-labelled S-HY was added to each well of ELISA plates and incubated for 2h at room temperature, the radioactivity remaining in each well after washes corresponded to 0.044±0.004% (n=3) of the added material. A 16 h

incubation period resulted in a binding of 0.076±0.023% of the material. In a similar experiment with another batch of ^{125}I-S-HY the presence of hUC-HA and S-HA reduced the binding from 0.02±0.005% to 0.008±0.007% and 0.007±0.006%, respectively (Fig 2).

As the low amount of binding to the plastic wells corresponded to the low amount of protein found in S-HY and as the S-HA and hUC-HA reduced the binding of the radioactivity it pointed to the presence of protein in S-HA and hUC-HA that bound to the plastic. By using a sensitive assay Applicant found that the hUC-HA contained 2.3% protein, the S-HA 0.4%, RC-HA 0.3% and S-HY 0.09% protein. That the protein in these preparations really bind to the plastic was confirmed by protein analysis of the material after consecutive adsorption to six wells. Each well being incubated with the material from the previous well for 30-60 min. This reduced the protein to 1.6%,0.03% and 0.09% for hUC-HA, S-HA and RC-HA, respectively. The protein in S-HY was reduced to below the detection limit of 0.03% and in a preparation of CSA that was found to contain 0.05% protein, the protein content could be reduced to 0.03%. The protein content in the CSC preparation was below the detection limit.

SDS-PAGE with silver staining confirmed the presence of proteins, that these could partially be removed by adsorption and that the ELISA plates bound the proteins that were predominantly high Mw in S-HA but more heterogenous with high, but predominantly low, Mw:s for the hUC-HA (Fig 3). Using monospecific polyclonal antibodies to human fibronectin, a significant ($p<0.001$, $n=3$) positive reaction was found on the material from hUC-HA adsorbed to ELISA plates (140±7 mOD at 450 nm) in relation to S-HA (63±2 mOD), that was not different from background level (53±2 mOD). The reaction on hUC-HA treated wells using preimmune serum (61±10 mOD) was not different from background but significantly different from the specific antibody ($p<0.001$, $n=3$). Using a competition ELISA, that is not yet fully developed, a preliminary

concentration of fibronectin in hUC-HA was found to be in the range of 0.05%. In immunoblotting experiments after electrophoresis of adsorbed hUC-HA material and using the polyclonal antisera to fibronectin, a positive reaction could be found at 220-240 kDa, corresponding to the double band produced by human fibronectin (Fig 3).

When ^{125}I-HA was added to fibronectin-coated ELISA plates, the amount of radioactivity that could be extracted from the wells after a 1-2h incubation (10±2 dpm) was not different from the background level of empty tubes used for counting (8±6 cpm).

Discussion

The above process could be made more efficient using e.g.. plastic beads in order to increase the "active" surface. The beads could be used for batchwise purification or placed in a column through which the preparation can be passed for a continuous purification. Also, HPLC (High Performance Liquid Chromatography) could be used as well, or any other suitable means. Physiological pH and ionic strength was predominantly used in this study because it was used in some previous studies, did not differ significantly from conditions used to adsorb proteins for ELISA, and also because the results then can be compared with experiments using living cells and animals. However, other pH and ionic strength values may be employed.

Autoradiography of SDS-PAGE gels used to separate material from adsorbed ^{125}I-S-HY showed that the adsorbed labeled material had a similar profile as the protein staining of adsorbed S-HY (not shown). The method of labelling (Gustafson et al, 1994 idem) would also label the protein in the preparation, and the reduced binding of radioactivity in the presence of hUC-HA and S-HA to levels around background (Fig 2), suggests that the unlabeled proteins in the preparations compete with the radiolabeled proteins for binding. This is also

likely to be the reason behind the finding that the labelled S-HY associated to fibronectin coated dishes is not significantly different from background.

The presence of fibronectin in hUC-HA was not too surprising. It can be expected that protein contaminants are from proteins normally found in connective tissue as such tissues are the source for the animal HA. The immunoblotting experiment revealed staining at 220-240 kDa as expected for fibronectin. However, the staining was not very prominent and staining at higher as well as lower Mw:s indicates that nonspecific staining may have occurred.

One preparation, S-HY could be purified by consecutive adsorption of proteins to ELISA plates to ultrapure material (UP-HY) where no protein could be detected (<0.03%).

As many changes can be made to the embodiments without departing from the scope of the invention, it is intended that all material contained herein be interpreted as illustrative of the invention and not in a limiting sense.

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE AS FOLLOWS:

1. A process of purifying a polysaccharide, said process comprising:

introducing the polysaccharide which contains contaminants onto a medium, which medium attracts the contaminants thereto, for a predetermined period of time; and recovering the contaminant reduced polysaccharide.

2. The process of Claim 1 further comprising the step below, before recovering the contaminant reduced polysaccharide which comprises:

transferring the contaminant reduced polysaccharide from the medium after said predetermined period of time and introducing said contaminant reduced polysaccharide from the medium to a second medium which second medium attracts contaminants and continuing said process until the desired degree of purification is achieved.

3. The process of Claim 1 or 2 wherein said medium comprises plastic.

4. The process of Claim 3 wherein said plastic is a plurality of plastic beads or the like.

5. The process of any of the preceding claims wherein said process is carried out at substantially physiological pH.

6. The process of any of the preceding claims wherein said process is carried out at substantially physiological ionic strength.

7. The process of any of the preceding claims wherein the polysaccharide, after being subjected to purification, is at least 99.91% free of contaminants.

8. The process of any of the preceding claims wherein said polysaccharide is a glycosaminoglycan.

9. The process of Claim 7 wherein the glycosaminoglycan is selected from hyaluronan and chondroitin sulphate.

10. The process of any of the preceding claims wherein the polysaccharide is selected from hyaluronan and chondroitin sulphate.

11. A highly purified form of a polysaccharide having a protein content no greater than about 0.09% by weight.

12. A highly purified form of a polysaccharide having a protein content no greater than about 0.03% by weight.

13. A highly purified form of a polysaccharide having a protein content no greater than 0.03% by weight.

14. The polysaccharide of Claim 10, 11 or 12 wherein said polysaccharide is selected from hyaluronan and chondroitin sulphate.

15. The polysaccharide of Claim 13 wherein said polysaccharide is hyaluronan.

16. A polysaccharide being at least 99.91% free of contaminants whenever prepared by the process of any of Claims 1-10.

17. Hyaluronan having a protein content no greater than about 0.09% weight whenever prepared by the process of any of Claims 9 or 10.

18. Hyaluronan having a protein content no greater than 0.03% by weight whenever prepared by the process of any of Claims 9 or 10.

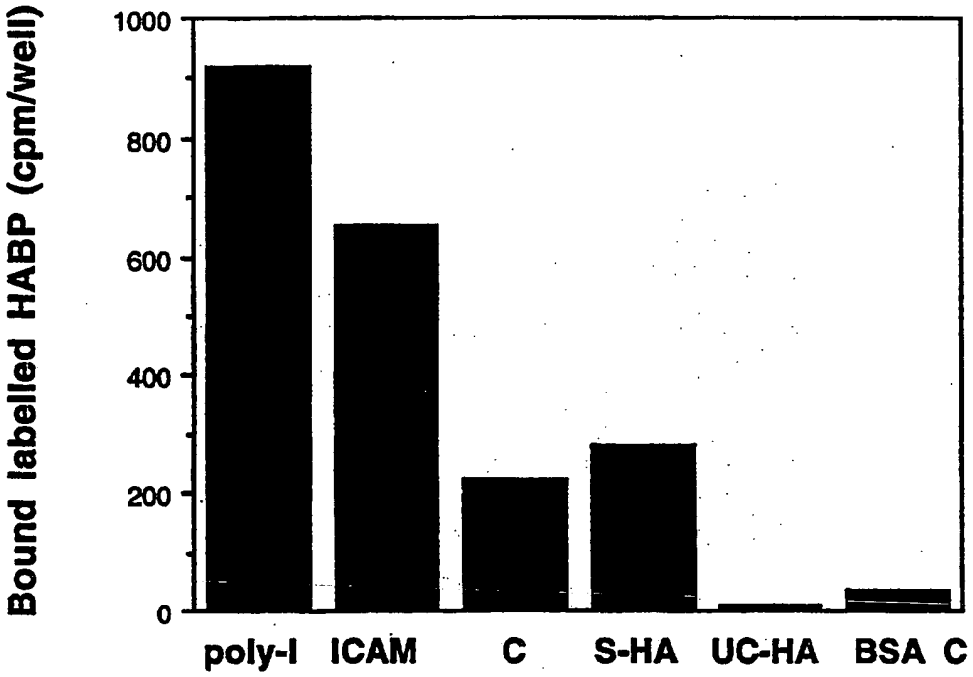
Bound labelled HABP (cpm/well)
1000
800
600
400
200
0
poly-I
ICAM
C
S-HA
UC-HA
BSA C

FIGURE 1

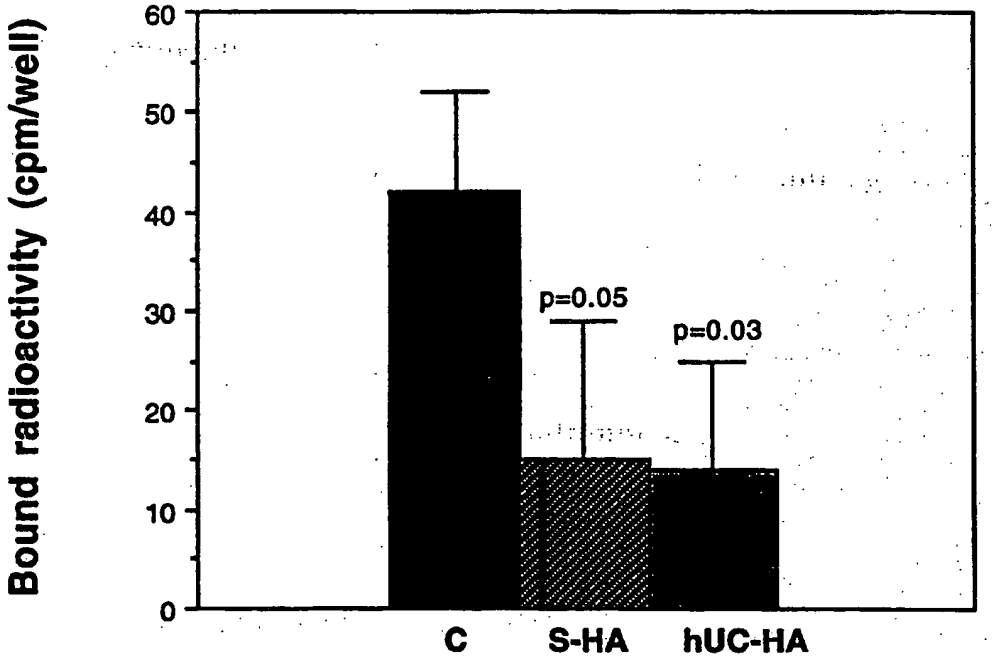
Bound radioactivity (cpm/well)
60
50
40
30
20
10
0
p=0.05
p=0.03
C
S-HA
hUC-HA

FIGURE 2

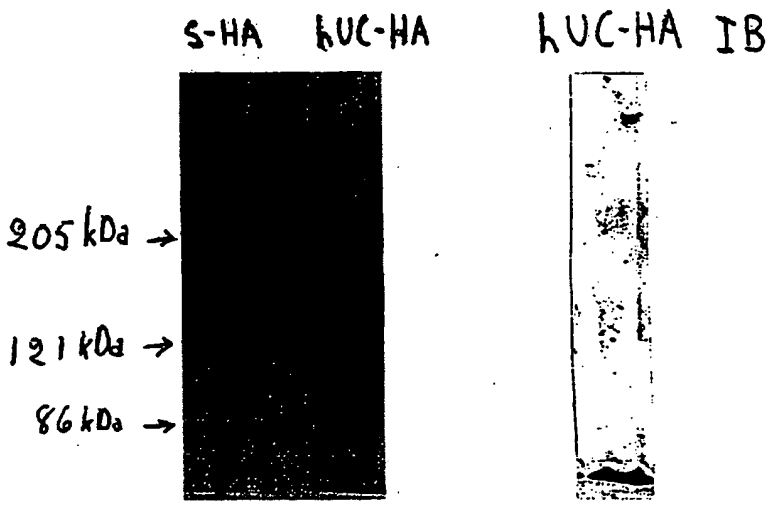

FIGURE 3